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Title: Life on the Edge: Microbes in Rock Varnish

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Abstract

The newly discovered high concentrations of manganese on Mars open up new possibilities for habitability on that planet [1, 2]. On Earth, there is a close association between Mn deposits and the presence of Mn- and Fe- oxidizing microbes [3-7]; as such, Mn is considered a principal biosignature for Mars [8]. The most common terrestrial Mn-rich surface material on Earth is desert varnish, a dark, shiny coating on rocks in arid locations. Microorganisms occupy various niches within rock varnish, and it is probable that the concentration of Mn in many rock varnishes is mediated by microbial activity [6, 9, 10]; however, the relationship between microbes and varnish remains a source of long-standing controversy [11]. Do microorganisms drive the formation of varnish, oxidizing Mn and Fe to gain energy, or do they instead utilize varnish as a habitat? This long-standing question has important implications for our understanding of how life on Earth has evolved to capture and harness energy from the physical environment. Additionally, it will greatly influence the search on Mars for the signatures of life that may be present in rock varnish cannot be definitively identified on Mars without first being identified on Earth. Here we explored the microbial species and processes involved in the habitation of rock varnish and identified organic biosignatures that, in concert with trace element and mineralogy, could be used to distinguish the biogenic and abiogenic origins of terrestrial Mn-rich surfaces so that we may then apply the knowledge gained in this work to current Martian datasets from ChemCam. Each of these goals has important implications for our understanding of how life on Earth has evolved to capture and harness energy from the physical environment. A combinatorial experimental approach, utilizing microscopy, high-throughput DNA sequencing, culturing and physiological assays, were used to characterize the microbial communities inhabiting varnished rocks across the Western U.S. Microbial communities inhabiting varnish were shaped by both location (larger contribution) and rock type. However, we found that the unique varnish environment selects for a core group of radiation- and desiccation-tolerant microorganisms across landscapes and rock types. The bacteria comprising this core group include a who's who of radiation resistant organisms including *Rubrobacter* spp., *Deinococcus* spp., *Hymenobaceter* spp., and *Chroococcidiopsis* spp. Among the fungi, potential lichen-forming members of the *Lecanoromycetes* and rock-inhabiting *Dothideomycetes* were common. Importantly, we found that two of the survival mechanisms employed by these microorganisms are likely key drivers of Mn oxide formation and the biogenesis of varnish. First, we showed that the key cyanobacteria inhabiting the varnish accumulate copious amounts of low molecular weight Mn^{2+}-conjugates, that are critical for their ability to withstand the toxic effects of reactive oxygen species generated upon radiation exposure. Secondly, the microbial community inhabiting varnished rocks were found to be particularly adept at producing siderophores, which are high-affinity chelating compounds secreted by bacteria and fungi that serve to sequester Fe^{2+} (and Mn^{2+}) from oxidizing environments for transport into cells as essential micronutrients for growth and survival. When tightly bound to organic ligands, Mn^{2+} is susceptible to oxidation to Mn^{3+}-ligand under atmospheric oxygen and at slightly basic pH levels. Further, Mn^{3+}-ligand can be oxidized to MnO_2 (manganese oxide) when it comes into contact with reactive oxygen species (e.g., superoxide radical) formed during the photolysis of water during periods of high UV exposure. The data points towards a scenario whereby cyanobacterial biofilms, and associated radiation resistant, heterotrophic bacteria, comprise the

central hub of the varnish community. These communities depend on light and water to slowly establish on the rock surface where they accumulate and concentrate Mn for survival. When the biofilms dry out, mineral rinds rich in Mn oxides form which serve as a habitat for the successive rounds of microbial growth and Mn oxide formation. Copious production and excretion of siderophores, which are extremely stable in the environment, coats the rock surface with a long-lived catalyst for Mn oxidation. By discovering these two completely novel mechanisms for biogenic varnish formation, we have gained the underlying knowledge necessary to develop a suite of organic and inorganic biosignatures to distinguish biogenic and abiogenic origins of Mn-rich surfaces on Earth or Mars.

Background and Research Objectives

Rock varnish: The ubiquitous desert rock coating.
Rock varnish is a thin (micron-scale), dark brown or black, often shiny layer on rock surfaces that forms in arid regions all over the Earth. Its dark appearance comes from the presence of Mn and Fe-oxides that make up ~30% of its composition [12]. Because it forms on rocks that are not necessarily Mn-rich, varnish is thought to be derived from materials external to the rock such as atmosphere, air-fall dust and surrounding soils [13-15]. Despite its ubiquity on the Earth, the formation mechanism of varnish is not well understood; although biotic, abiotic and paired biotic/abiotic formation mechanisms have been proposed [16], the concentration of Mn appears to be mediated by microbial activity in many cases [6, 9, 10].

Microbial processes: A window into early terrestrial life?
Several lines of evidence suggest that microorganisms capable of oxygenic photosynthesis (where water serves as the electron donor yielding O_2; the type of photosynthesis carried out by most cyanobacteria, algae and land plants) are not suited to flourish in mature rock varnish [16, 17]. Rather, existing research points towards a scenario in which the highly oxidative, Fe/Mn-rich varnish environment favors an oxygenic autotrophy (i.e., Fe^{2+} and Mn^{2+}, as electron donors) and which would require the establishment of highly specialized species. Recently, a radical new mechanism by which microorganisms could harvest energy from sunlight was proposed, but not empirically tested, by Nealson [18]. In this process, called Ex-phot, thinly layered Mn/Fe oxides in rock varnish undergo photo-reduction during daylight hours, and then serve as a source of reductant for resident microorganisms that are capable of oxidizing Fe^{2+} or Mn^{2+}. If the energy gained from this light-dependent Mn redox cycle is sufficient to support CO_2 fixation, Ex-phot would embody a completely novel form of phototrophy capable of sustaining life in the extreme varnish environment. This hypothesis builds upon work suggesting that a biologically-linked Mn cycle predated the oxidation of Earth's atmosphere and was the precursor to the Mn_4CaO_5 cluster that catalyzes water oxidation in modern day photosystem II [19]. Additionally, Mn oxides have recently become a hotbed of activity in the quest to design a bioinspired solar water-splitting technology for clean energy [13, 20]. Thus, the work proposed here has the potential to completely reshape the current understanding of pre-photosynthetic modes of light utilization and will advance the field of artificial photosynthesis.

Rock varnish on Mars?

Observations on Mars of dark, shiny rocks that appear similar to varnished surfaces have led to the question of whether Mn-enriched coatings could have formed on Mars [21-24]. Although iron oxides such as magnetite and hematite have been observed on the martian surface both from orbit and in situ [25, 26], there has been no similar widespread observations of Mn-oxides from orbit. However, recent in situ observations from the Curiosity rover have discovered a wealth of high-manganese materials in Gale crater, some of which appear as Mn-oxide layers [1, 2]. It should be noted that the X-ray diffraction instrument onboard Curiosity, CheMin, cannot analyze a thin surface layer due to the constraints of the rover drill and sampling mechanism. Only material deeper than ~1.5 cm within the drill hole is delivered to the CheMin instrument [27]. However, the upcoming Mars 2020 rover will include two Raman instruments (the LANL-led Super-Cam and SHERLOC) that will provide detailed mineralogical characterization of rock surfaces. In order to identify Mn-related biosignatures on Mars, it is of the utmost importance that these biosignatures be identifiable with rover payload instruments such as ChemCam & SuperCam. The work proposed here will address that knowledge gap, generating a database directly linking ChemCam & SuperCam data to specific molecular structures and terrestrial biosignatures.

Scientific Approach and Results

Project Goal 1: Characterize the rock varnish microbial community. We examined rock varnish at the microscale using SEM to better understand its spatial distribution and structure along the rock surface. Synchotron XRF and XAS analysis was applied to the same samples to assess varnish mineralogy. The microbial community in varnish samples were analyzed using metagenomics (16S rRNA/ITS amplicon and shotgun libraries) and fluorescence microscopy to 1) determine the identity of the "core" microbiome present in rock varnish, and 2) gain insight into the metabolic processes carried by microorganisms inhabiting the varnish. Rock varnish samples were collected from 7 locations (AZ, NM, UT and WY) and consisted of five underlying rock types (basalt, rhyolite, tuff, sandstone, sedimentary conglomerate) (Figure 1). Microscopic analysis of varnish thin sections revealed rock surfaces with discontinuous patches of laminated varnish that ranged in thickness from several nm to ~100 nm. Cell-like structures were observed rarely (e.g., light micrograph; Figure 2, Panel A). Pinnacles were observed

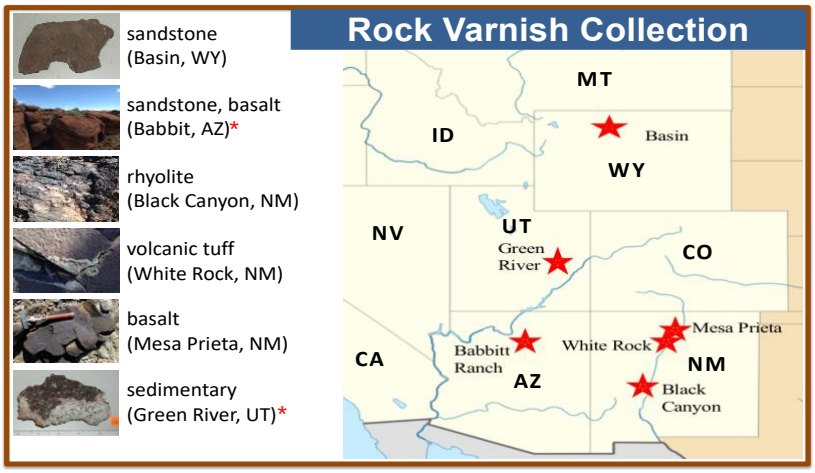

Figure 1. Rock varnish sample suite

in many varnish samples (e.g., SEM images for rhyolite shown in Figure 2, Panels B, C and basalt shown in Panel D) that are reminiscent of light-dependent growth patterns observed in microbial structures such as stromatolites or other biofilms. Pinnacled growth at different scales were observed (Panel C) and in some instances the "valleys" between varnish pinnacles appeared to be filled with detritus (Panel D). These patterns were observed in the majority of samples visualized, indicating that light plays a role in varnish formation at the micro-level, as has been previously suggested [14] and long suspected based on macro-level observations of varnish presence/absence on different rock faces (i.e. facing towards or away from direct sunlight) [4].

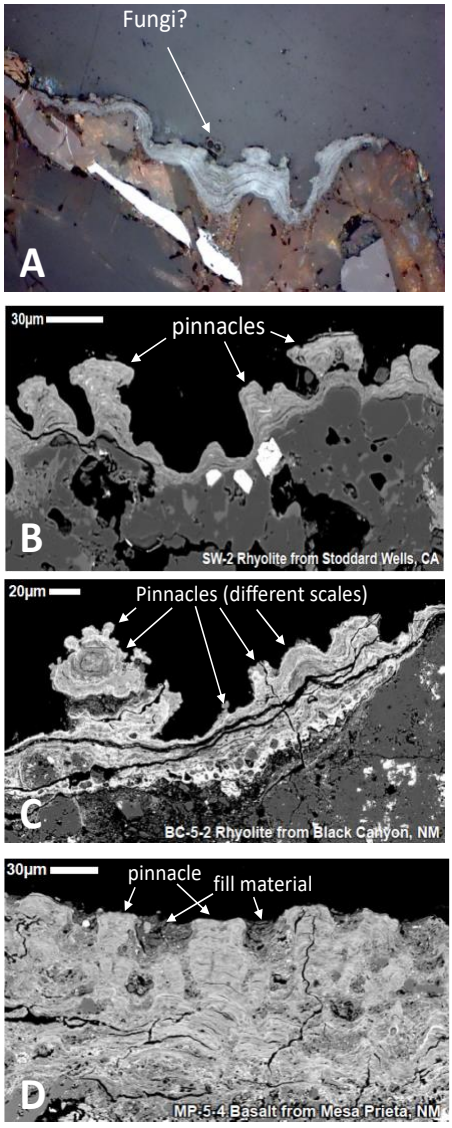

Figure 2. Microscopic analysis (A, light microscopy; B-D, scanning electron microscopy) of select rock varnish samples.

Mn concentrations and redox speciation were mapped within the rock thin sections using synchrotron XRF. Mn was concentrated within the varnished regions, particularly within pinnacled structures (Figure 3A, B). The preponderance of Mn within the varnish region of the thin sections was in the form of Mn(IV), yet redox mapping also identified zones of varnish containing Mn(III) in many samples. In several instances (particularly within the pinnacled structures), the relative proportion of Mn(III) was nearly equal to that of Mn(IV) (Figure 3C). Mn(II) was not detected in varnished regions, but igneous Mn(II) was often detectable in the underlying rock (Figure 3C). Synchrotron XAS spectra of a series of points in several thin sections overlying regions of Mn(III) as detected by XRF, confirmed the presence of Mn(III) (e.g., Figure 3D). The median Mn k-edge spectrum of the rock varnish samples analyzed (n=6) fits to 69% Mn(IV) and 31% Mn(III) (data not shown). **This is the first confirmation of Mn(III) within rock varnish.** The presence of regions of Mn(III) mixed in with Mn(IV) provides a strong argument for redox cycling of Mn within the varnish or for the incorporation of Mn(III) into the crystal lattice of the varnish during its formation. These observations are critical for understanding how trace elements such as zinc, lithium, barium, etc. are incorporated into the lattice of Mn oxides found in biogenic rock varnish, with implications for interpreting the elemental composition of high Mn materials in existing and future ChemCam LIBS datasets from Mars (see discussion of siderophores under the *Project Goal 2* section).

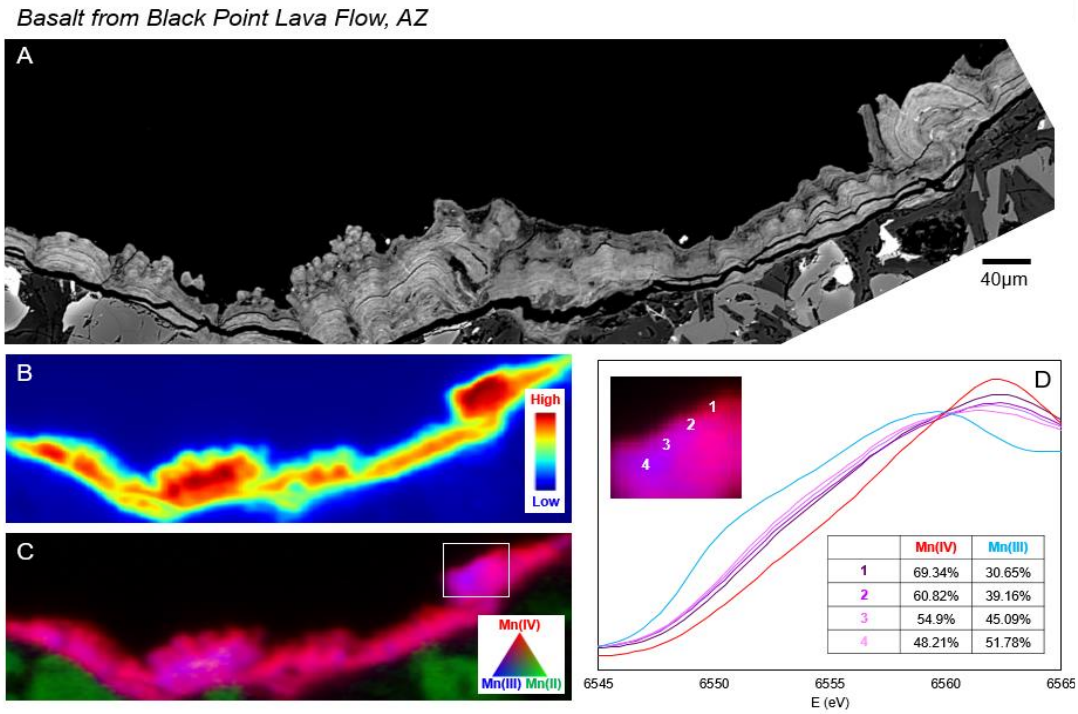

Figure 3. Images of varnished basalt from Black Point Lava Flow, Babbitt Ranches, AZ. A) SEM image showing the stratified, thin layers of varnish on the rock surface (note pinnacled structures). B) Synchotron XRF map showing Mn abundance. C) Synchrotron multiple energy map showing redox speciation of Mn. D) Point spectra from within C (inset), corroborating the presence of the Mn redox gradient. Relative contribution of Mn(III) vs. Mn(IV) was quantified by least squares fitting.

Amplicon libraries targeting the 16S rRNA gene for bacteria and the ITS region for fungi were prepared and sequenced for each of the samples (~65 rock varnish samples, 19 soil samples). Varnish communities were significantly different from surface soil communities, particularly the fungal communities (Figure 4). Microbial communities inhabiting varnish were shaped by both location (larger contribution) and rock type, but we found that the unique varnish environment selects for a core group of radiation- and desiccation-tolerant microorganisms that exists across landscapes and rock types (Figure 5). Dominant sequence types detected for both bacterial and fungal sequences are from taxa that typically inhabit environmental surfaces exposed to high sunlight – particularly stone. The taxa detected in the varnish communities represent a who's who of radiation resistant microbes, including bacterial members of the *Rubrobacter*, *Deinococcus*, *Hymenobaceter*, and *Chroococcidiopsis* [28, 29]. Fungal members of the Lecanoromycetes (often lichenized) and rock-inhabiting Dothideomycetes were also common, but on rock varnish without visible traces of lichen they typically comprised a minority of the microbial community (<10%). Common fungal taxa found associated with rock varnish in this study have been previously reported to be the dominant fungal inhabitants of cryptoendolithic niches (inside rocks) in Antarctica deserts and rocks in alpine environments [30, 31]. Representatives species of the *Chroococcidiopsis* and rock-inhabiting black fungi,

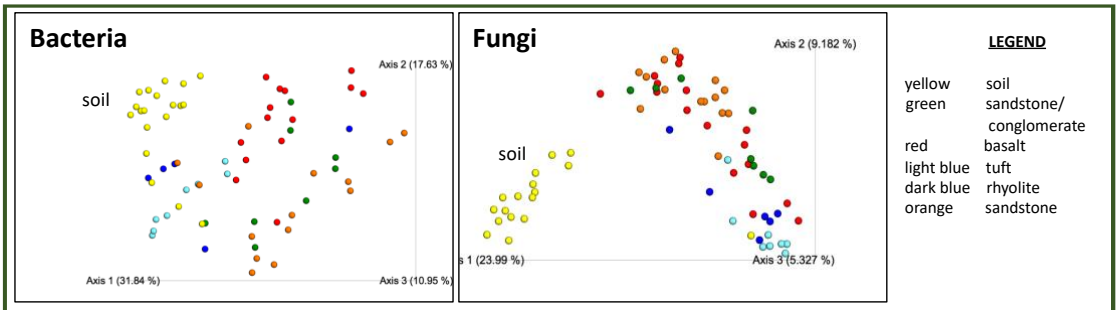

Figure 4. PCA plot of rock varnish bacterial and fungal communities demonstrating that the communities inhabiting the varnish across all sites and rock types were significantly different from surface soil communities, particularly among the fungi samples.

(*Cryomyces* spp.) have both survived treatments simulating Martian conditions on board the International Space Station [32, 33].

In particular, members of the cyanobacterial family Xenococcaceae (of which *Chroococcidiopsis* is a member) were found to be the dominant core member of the varnish community across locations and rock types, comprising approximately 30% of the community composition, on average (Figure 6A). *Chrococcidiopsis* is a member of the Xenococcaceae family of Cyanobacteria, which grow as freely aggregated cells in irregular, subspherical clusters or in colonies, attached to surfaces. Images of varnish flakes using confocal microscopy identified Xenococcaceae-like morphotypes (clusters of coccoidal cells) on the surface and embedded within the rock matrix (Figure 6B, C, D), providing evidence that these phototrophs are actively inhabiting the surface of varnished rocks, rather than dust-associated transients. Based on the relative abundance of Xenococcaceae sequence-types among the rock varnish libraries, the detection of highly similar sequences on rock varnish from 3 continents among multiple other studies [6, 9, 16, 34, 35], microscopic evidence, the proclivity of these cyanobacteria to inhabit rock crevices, cracks and fissures [36], we posit that they are a keystone species on rock varnish. Colonization by this hardy phototroph would enrich microenvironments on the rock surface with available C in the form of photosynthate and extracellular polysaccharides (Figure 6B), thus attracting mutualistic heterotrophs. Polysaccharides sheaths from the cyanobacteria could also serve as an attachment anchor and desiccation protectant for other colonizing microbes. Pigments, osmoprotectants and antioxidants produced by Xenococcaceae species and other early colonizers (see metagenomics results below) would aid in further community development.

Fungi	P-value
Lecanoromycetes	3.40^{-17}
Ascomycota	3.28^{-11}
Lecanorales	2.09^{-7}
Curvularia	6.94^{-6}
Eurotiomycetes	1.47^{-4}

Bacteria	P-value
Chroococcidiopsis	6.90^{-10}
Sphingomonas	1.94^{-8}
Xenococcaceae	2.12^{-8}
Propionibacteriaceae	4.64^{-7}
Xenococcaceae_1	4.57^{-7}

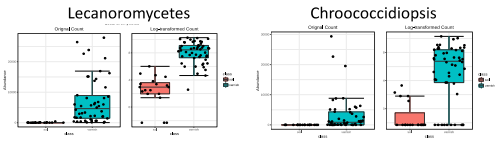

Figure 5. Examples of taxa common to varnish across different sites and rock types, but significantly less abundant in surrounding surface soils. Blue bars, rock varnish samples. Orange bars, soil samples.

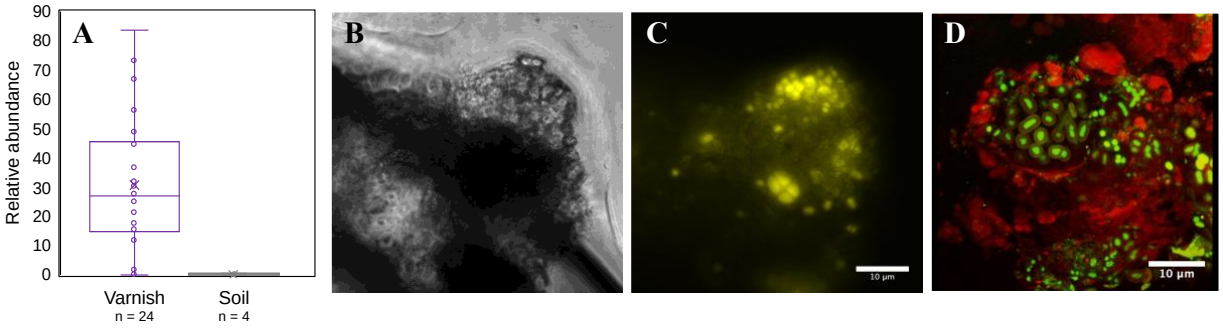

Figure 6. *Xenococcaceae/Chroococcidiopsis* sequences as a fraction of the total microbial community across 24 rock varnish samples versus that found in 4 control soil samples (A). Aggregated, coccoidal *Chroococcidiopsis*-like cell morphotype on rock varnish surface visualized by light microscopy (B) and fluorescence microscopy with the DNA-binding stain acridine orange (C, D). Note presence of polysaccharide-rich sheath surrounding cells in D.

Project Goal 2: Identify key organic signatures. We sought to identify unique and/or important organic biosignatures present in varnish. To do this, we characterized the organic materials extracted from fresh and previously collected rock varnish samples using a novel LESA LC-MS system developed expressly for this project (Figure 7). From a single varnished rock, 3,878 features were identified representing approximately 2,300 compounds/metabolites. Among these, less than 600 could be matched to a particular compound. Over 150 metabolic pathways were represented in the metabolites that were identified. Metabolites of interest included glutathione disulfide (a key compound for protection against reactive oxygen species), various photosynthetic electron carriers and structural units (e.g., Pheophytin b), and small organic molecules (e.g., oxalic acid, tartaric acid, etc.) that could serve as metal chelators/weathering agents.

As a proof of concept for the method, we have measured metabolites within, adjacent and far from a variety of lichen colonies growing on the surface of a varnished piece of volcanic tuff (Figure 8). A total of 16 sites on the rock varnish were targeted for extraction using deionized water. The Compoun d Discoverer software detected 683 features, representing 336 compounds, across all samples. Of particular note, sixteen of these

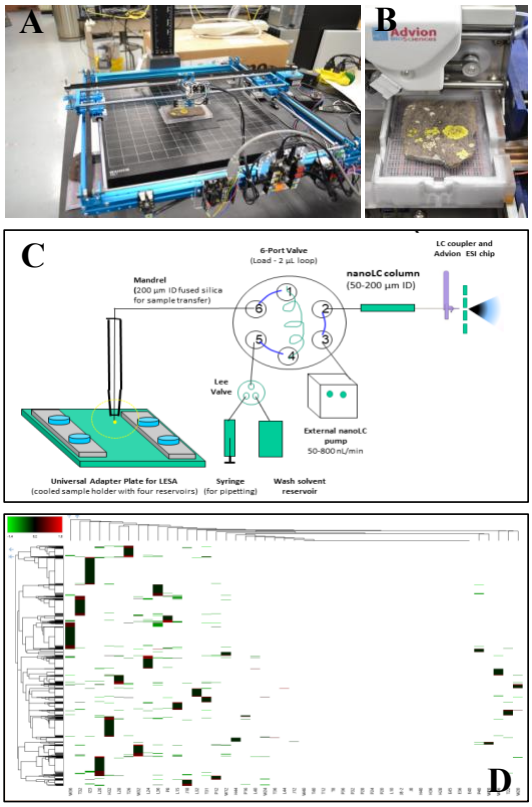

Figure 7. Automated LESA LC-MS system developed to analyze organics from rock varnish samples. A) A robot was developed to map the 3D surface of varnished rocks at a sub-mm level. B) These coordinates were used to guide the Avion Nanomate LESA+ ESI capillary tube to the rock surface to apply, then remove, solvents to collect organic molecules. C) The solvent was robotically injected into Thermo's Q Exactive™ Orbitrap Mass Spectrometer, D) and the resulting data was analyzed using Thermo's Compound Discoverer software and database.

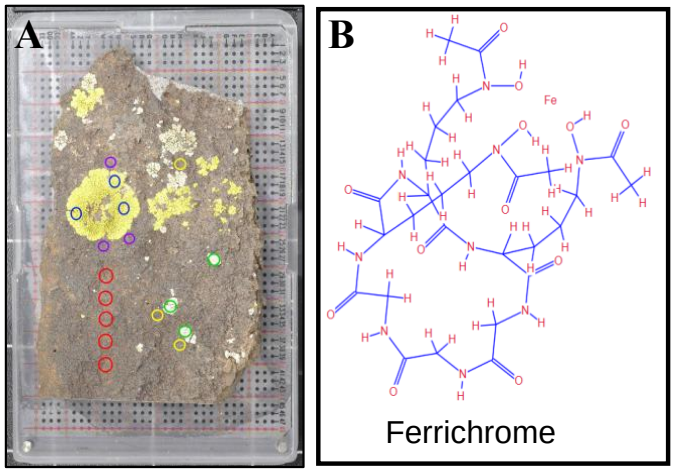

Figure 8. A) LESA LC-MS was used to examine the distribution of organic compounds on a lichenized piece of volcanic tuff. B) Structure of the siderophore, ferrichrome, which was detected on and near the yellow lichen.

compounds were categorized as Fe- or Mn-binding candidates based on predicted chemical formulas and diagnostic isotope patterns [37]. One compound was confidently identified as Ferrichrome (Figure 8B), with a 96.8% match to the authentic compound. Ferrichrome was detected at highest frequency on the surface of the yellow lichen colony. The detection of Ferrichrome and other siderophores on the surface of varnished rocks could has important ramifications for our understanding of rock varnish formation and the search for biosignatures associated with high concentrations of Mn oxides on the surface of Mars. Siderophores are chemically diverse secondary metabolites that primarily assist bacteria, fungi and plants to chelate iron [38, 39]. Siderophores are also capable of binding other metals, including Mn [38]. Indeed, binding coefficients for Mn by several siderophores are nearly equal to, or even greater, than that for Fe [40, 41]. Since varnished rock surfaces are one of the most oxidizing environments on the planet, the soluble forms of Fe and Mn required by microbes will be in scant supply on the surface of varnished rocks. Thus, they will depend on efficient chelators to scavenge both. The literature has clearly shown that once bound to a tightly binding ligand, Mn easily oxidizes from Mn(II) to Mn(III) and that Mn(III)-ligands can disproportionate to form Mn oxides [42, 43]. Additionally, ligand-Mn(II/III) complexes are more readily oxidized to Mn(IV) by superoxide [44], which will be produced on the surface of varnished rocks via photolysis of water.

We have found that the majority of bacterial and fungal isolates that we were able to culture from rock varnish were capable of producing strong metal chelators that acted on both Fe and Mn as visualized by CAS-agar assays. Additionally, metagenomic analysis of the gene content of the microbial community inhabiting the surface of two varnished rocks (basalt and sandstone) versus that of the microbial community associated with the non-varnished, underside of the same rocks reveals that the varnish communities invest more resources in order to scavenge iron and other metals. The percentage of genes devoted to transport of iron and siderophore metabolism were 79% and 119% greater, respectively, in the varnish communities. Overall, these results indicate that siderophores and other strong metal chelators, which are produced by the varnish community, may promote Mn oxide formation on rocks. Because of their role as an extracellular scavenger, siderophores are long lived in the environment and have been suggested as a potential biosignature [45]. Siderophores have also been shown to weather biogenic Mn oxides (i.e. birnessite) [46], and can preferentially remove Mn(III) from the Mn oxide lattice [46, 47]. Depending on the chemistry of the rock surface (rock mineralogy, wet/dry

atmospheric deposition, biological activity), different trace elements will replace the Mn(III) scavenged by siderophores, potentially yielding a signature for this process [47, 48].

Project Goal 3: Link environmental conditions, microbial activity, and Fe/Mn redox chemistry. The taxonomic identity of the microbial inhabitants of the rock varnish community identified in Goal 1 led us hypothesize that Mn was being concentrated by cells as defense mechanisms against reactive oxygen stress brought about by UV radiation. Although we identified extracellular Mn(II) oxidation in some fungi and a small sub-set of bacteria strains isolated from rock varnish (Figure 9), this phenotype was not detected in the many of the dominant rock varnish taxa determined by DNA sequencing. Additionally, metagenomic analysis of the rock varnish community did not reveal an enrichment of multicopper oxidase genes or NADH oxidases (responsible for extracellular Mn(II) oxidation in model organisms) or genes encoding for Mn transport compared to material scraped from the non-varnished surface (underside) of the same rocks (data not shown). On the other hand, a variety of genes involved in protection against reactive oxygen stress were found enriched in the rock varnish samples. For example, sequences involved in protection against oxygen stress and oxygen and light sensors were 60% and 401% more abundant in the varnish samples, respectively. Some of the critical responses of cyanobacteria to oxidative stress include the production of glutathione peroxidase-like proteins, glutathione-S-transferases and glutaredoxins, and non-enzymatic antioxidants including reduced glutathione, carotenoids, α-tocopherols and ascorbic acid [49, 50]. Table 1 shows the percentage of sequences coding for enzymes and metabolites involved in antioxidant activity in metagenome libraries prepared from scrapings obtained from the varnished versus unvarnished surface of two rocks.

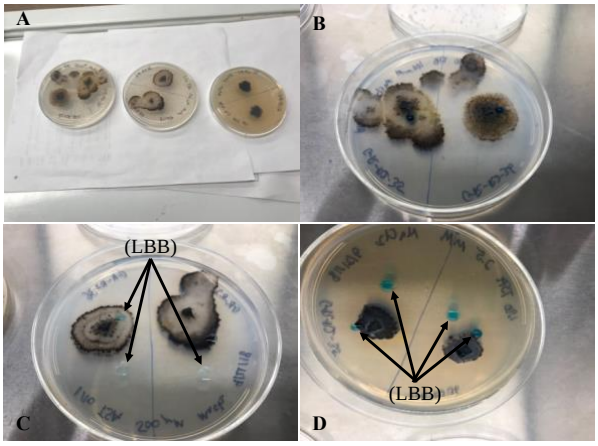

Figure 9. Mn(II) oxidation by fungal strains isolated from rock varnish. The dark coloration of fungal hyphae is due to melanin-based pigments for UV protection. Black granules are likely Mn oxide precipitates, based on Leukoberbelin blue (LBB) assays (blue droplets in or immediately adjacent to colonies; C, D). Brown discoloration of the medium (D) indicates the presence of Mn oxides formed via a diffusible substance from the fungal colonies, which was confirmed by the LBB assay (blue droplets in D separate from fungal colonies).

Subsystem category	% of all classified reads				enrichment in varnish (fold)
	varnished		unvarnished		
	sandstone	basalt	sandstone	basalt	
Oxidative Stress level II	1.262	1.187	0.980	0.892	1.31
Oxidative Stress level III	0.328	0.229	0.205	0.190	1.41
Protection from ROS	0.090	0.086	0.064	0.046	1.60
Superoxide dismutase	0.016	0.039	0.021	0.022	1.30
Catalase	0.072	0.074	0.047	0.029	1.92
Glutathione S-transferase	0.164	0.102	0.048	0.031	3.36
Glutathione reductase	0.053	0.084	0.013	0.004	7.83
Rubreythrin	0.069	0.064	0.042	0.044	1.54
Ferroxidase	0.009	0.018	0.004	0.003	3.91
Alkyl hydroperoxide reductase	0.052	0.038	0.024	0.023	1.90
Organic hydroperoxide resistance protein	0.012	0.009	0.005	0.007	1.75
Phytochelatin synthase	0.004	0.002	0.001	0.000	4.82

Table 1. Relative abundance of sequences encoding genes involved in oxidative stress recovered from the varnished versus unvarnished surface of basalt and sandstone

Recent research has revealed that Mn(II) plays a critical role in survival of many radiation-resistant microorganisms to ionizing

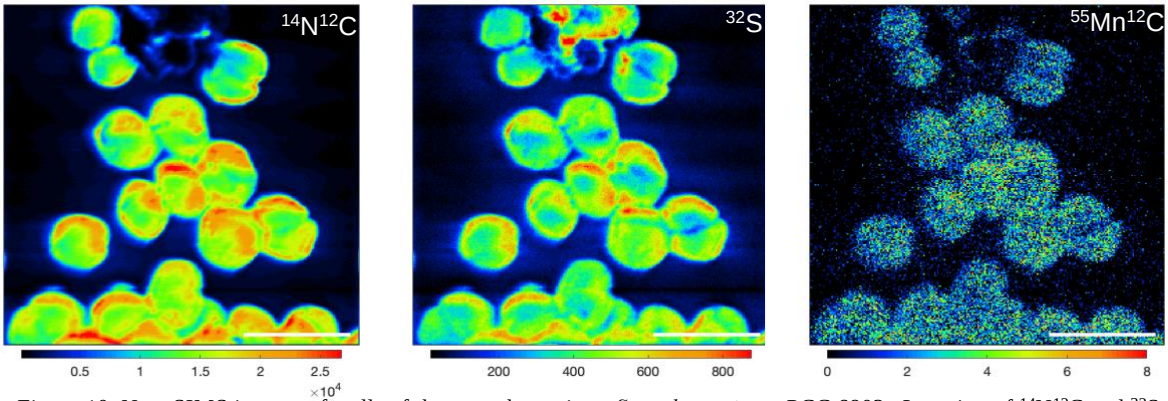

Figure 10. NanoSIMS images of cells of the cyanobacterium, *Synechocystis sp.* PCC 6803. Location of $^{14}N^{12}C$ and ^{32}S are compared to that of $^{55}Mn^{12}C$ to demonstrate that Mn(II) is internalized and distributed equally throughout the cell.

radiation. Using the radiation-resistant model organism, *Deinococcus radiodurans*, Daly et al. [51] discovered this organism hoards substantial amounts of Mn(II) compared to the typical bacteria, and that intracellular Mn(II) ions in high-symmetry antioxidant complexes with small molecules (othrophosphate, peptides) protect cells, particularly their proteins, from reactive oxygen species formed upon irradiation. A follow-up study found that across the tree of life, radiation resistance is best predicted by the cellular content of these Mn-small molecule complexes [52]. Because the rock varnish community was found to be comprised of many microbial taxa known to be resistant to radiation, we set about to determine the extent that rock varnish microorganisms could accumulate Mn(II), with a focus on cyanobacteria, including *Chroococcidiopsis*. Attempts to culture *Chroococcidiopsis* from varnish material proved unsuccessful, so we ordered a strain that had been previously isolated from a dried pool (*Chroococcidiopsis* sp. PCC 7433) and another strain (*Chroococcidiopsis* sp. Ryu 1-3; isolated from a stone bench in Japan), whose 16S rRNA molecule exhibited 100% similarity to that of the most abundant *Chroococcidiopsis* sequence-type in our varnish libraries. All cyanobacteria tested and accumulated high amounts of Mn(II). NanoSIMS was used to visualize the location of the internalized Mn, and it was found to be distributed throughout the cell (Figure 10). Next, electron paramagnetic resonance (EPR) spectroscopy was used to demonstrate that Mn(II) is largely associated with low molecular weight species in the cell, rather than bound to larger molecules such as proteins (data not shown). Variations of electron-nuclear double resonance (ENDOR) spectroscopy were then used to determine that the Mn(II) accumulated by cyanobacterial cells is most likely associated with carboxylate ligands (Figure 11), which is different from *Deinococcus*, other bacteria and green algae, where Mn(II) is associated with nitrogen-ligands in small peptides and phosphate-ligands in inorganic phosphate [51-53]. These results provide that first evidence that cyanobacteria, including the extreme radiation-resistant genus *Chroococcidiopsis*, accumulate Mn as an antioxidant to protect against reactive oxygen species assault on cells that occur during desiccation or UV exposure.

The combined action of siderophores and the Mn accumulating phenotype observed across radiation resistant bacterial taxa [52], including those that are abundant members of the varnish community (e.g., *Rubrobacter*, *Deinococcus*, *Chroococcidiopsis*), provide a biological mechanism whereby Mn is concentrated relative to other elements on the surface of rocks over

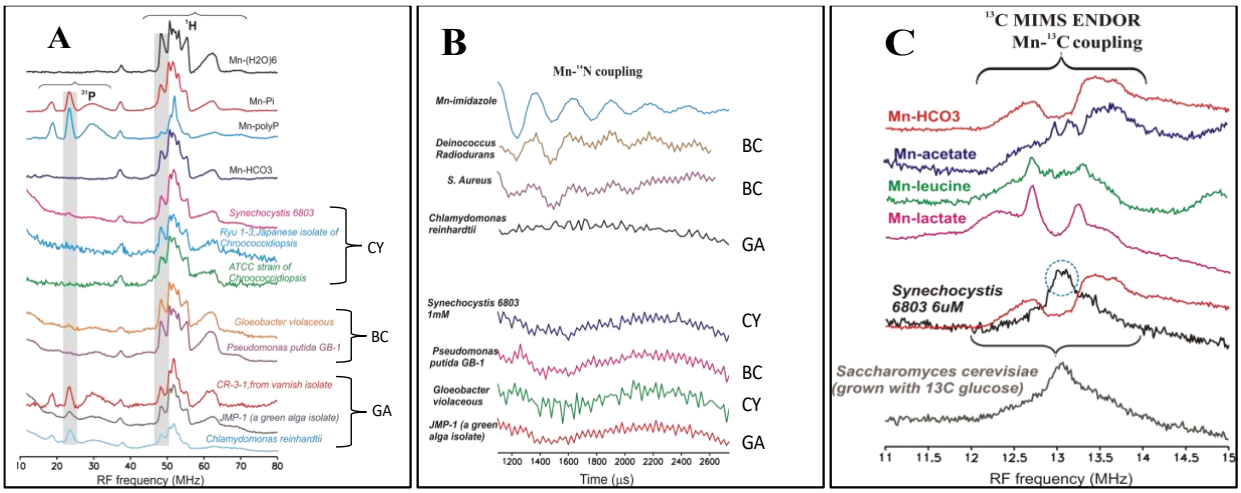

Figure 11. ENDOR analysis of Mn(II)-loaded cells. A) Davies ENDOR spectra show cyanobacterial Mn(II) is bound to some non-water, non-phosphate ligands, whereas algal Mn(II) shows a significant phosphate ligand component. B) 3-pulse EESM spectra show no detectable Mn(II) bound to N ligands. C) MIMS ENDOR with ^{13}C label shows Mn(II) in the cyanobacteria *Synechocystis sp.* PCC 6803 is most likely bound to carboxylate ligands.
Abbreviations: cyanobacteria, CY; bacteria, BC; green algae, GA

time. When conditions are good (i.e. wet) biofilms will form on the surface of rocks, with *Chroococcidiopsis* acting as a keystone member of the community, providing fixed C and attachment sites for other organisms. Even during wet periods (years, decades, centuries), these microorganisms are exposed to excessive oxidative stress imposed by hourly or daily dry/wet cycles and unrelenting exposure to UV. Thus, any available Mn or the rock surface, whether from weathering of the underlying rock (minimal input) or wet/dry atmospheric deposition (primary source), is a precious resource for these organisms and will be hoarded. When climatic conditions are too adverse, the microbial community will collapse and mineral rinds rich in Mn oxides form which will serve as a habitat for the successive rounds of microbial growth (wet periods) and Mn oxide formation (dry periods).

Anticipated Impact on Mission. Characterization of the microbial community inhabiting rock varnish and the physiological traits that enable them to inhabit one of the most oxidizing environments on the planet has several impacts on LANL's mission. First, the results highlight the importance of manganese in radioresistance among prokaryotes in the environment and the identification of carbonyl-Mn(II) ligands could inform the development of radiation countermeasures [54, 55],addressing needs in national security. Second, we now have the underlying knowledge necessary to develop a suite of organic and inorganic biosignatures to distinguish biogenic and abiogenic origins of Mn-rich surfaces on Earth or Mars. This work advances LANL's Science of Signatures Pillar. We will submit a proposal to NASA's Exobiology program to determine if trace elements such as zinc, lithium, barium, etc. that are incorporated into the lattice of Mn oxides found in rock varnish can be definitively treated as a biosignature for siderophore weathering.

Conclusion. A core group of microorganisms were found to inhabit rock varnish across the Western U.S., which include bacterial species among the *Rubrobacter*, *Deinococcus*, *Hymenobaceter*, and *Chroococcidiopsis* genera and fungal members of the *Lecanoromycetes* and rock-inhabiting *Dothideomycetes*. Importantly, we found that two of the survival mechanisms employed by these microorganisms are likely key drivers of Mn oxide formation and the biogenesis of varnish. First, we showed that the key cyanobacteria inhabiting the varnish accumulate copious amounts of low molecular weight Mn^{2+}-conjugates, that are critical for their ability to withstand the toxic effects of reactive oxygen species generated upon radiation exposure. Secondly, the microbial community inhabiting varnished rocks were found to be particularly adept at producing siderophores, which are high-affinity chelating compounds secreted by bacteria and fungi that serve to sequester Fe^{2+} (and Mn^{2+}). When tightly bound to organic ligands, Mn^{2+} is susceptible to oxidation to Mn^{3+}-ligand under atmospheric oxygen and at slightly basic pH levels. Further, Mn^{3+}-ligand is readily oxidized to MnO_2 (manganese oxide) when it comes into contact with reactive oxygen species (e.g., superoxide radical) formed during the photolysis of water during periods of high UV exposure. The data points towards a scenario whereby cyanobacterial biofilms comprised primarily of *Chroococcidiopsis* spp., and associated heterotrophic bacteria comprise the central hub of the varnish community. These communities depend on light and water to slowly establish on the rock surface where they accumulate and concentrate Mn and produce large amounts of extracellular siderophores. When the biofilms dry out, mineral rinds rich in Mn oxides form which serve as a habitat for the successive rounds of microbial growth and Mn oxide formation. Additionally, production and excretion of siderophores, which are extremely stable in the environment, coats the rock surface with a long-lived catalyst for Mn oxidation. By establishing these two completely novel mechanisms for biogenic varnish formation, we have gained the underlying knowledge necessary to develop a suite of organic and inorganic (trace elemental composition of Mn oxides formed in the presence of siderophores) biosignatures to distinguish biogenic and abiogenic origins of Mn-rich surfaces on Earth or Mars.

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